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(71) Applicant: SMITHKLINE BEECHAM PLC [GB/GB]; New Horizons Court, Brentford, Middlesex TW8 9EP (GB).

(72) Inventors: MACPHEE, Colin, Houston; SmithKline Beecham Pharmaceuticals, New Frontiers Science Park South, Third Avenue, Harlow, Essex CM19 5AW (GB). MOORES, Kitty; SmithKline Beecham Pharmaceuticals, New Frontiers Science Park South, Third Avenue, Harlow, Essex CM19 5AW (GB). BERKHOUT, Theodorus, Antonius; SmithKline Beecham Pharmaceuticals, New Frontiers Science Park South, Third Avenue, Harlow, Essex CM19 5AW (GB).

(74) Agent: CONNELL, Anthony, Christopher; SmithKline Beecham, Corporate Intellectual Property, Two New Horizons Court, Brentford, Middlesex TW8 9EP (GB).

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(57) Abstract

The ligand PF–4 has been identified as a ligand for the 7TM receptor HBMBU14, also known as TYMSTR, STRL–33 and BONZO.

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PF-4 RECEPTOR ASSAY

This invention relates to the identification of the ligand for an orphan 7TM receptor and its use as a screening tool to identify antagonists and agonists of the receptor, to the antagonists and agonists so identified and their use in therapy.

Chemokines are structurally and functionally related 8 to 10 kD polypeptides, involved in the recruitment of white blood cell into areas of inflammation and their subsequent activation (Miller, M.D. and Krangel, M.S. (1992) *Crit. Rev. Immunol.* **12**, 17-46; Baggiolini, M., Dewald, B. and Moser, B.(1994) *Adv. Immunol.* **55**, 97-179). In addition, some chemokines are able to regulate the proliferative potential of hematopoietic progenitor cells, endothelial cells and certain types of transformed cells (Oppenheimer, J.J., Zachariae, C.O.C., Mukaida, N., and Matsushima, K. (1991) *Ann. Rev. Immunol.* **9**, 617-648; Schall, T.J. (1991) *Cytokine* **3**, 165-183). Based on whether the first two cysteine moieties are separated by one amino acid residue or are adjacent, chemokines belong to the α- or CXC chemokine family (e.g interleukin (IL)-8) or the β- or CC chemokine family (e.g. RANTES and MCP-1). CXC chemokines play a key role in the accumulation of various cell types, including neutrophils, monocytes, T-lymphocytes, basophils and fibroblasts at sites of inflammation. These chemokines are implicated in both acute and chronic inflammatory disease states, including rheumatoid arthritis, inflammatory bowel disease, atherosclerosis, asthma, leprosy, psoriasis, various respiratory syndromes, and also contribute towards modulation of angiogenesis and fibroplasia.

Chemokines express their biological responses through interaction with chemokine receptors (Horuk, R. and Peiper, S.C. (1995) *Exp. Opin. Ther. Patents* **5**, 1185-1200).

Several chemokine receptors have already been cloned, for instance, the following human CXC chemokine receptors:
the receptors for IL8 (CXCR1) and IL8/ELR chemokines, (CXCR2, Holmes, W.E., Lee, J., Kuang, W.J., Rice, G.C. and Wood, W.I. (1991) *Science* **253**, 1278-1280; Murphy, P.M. and Tiffany, H.L. (1991) *Science* **253**, 1280-1283);
a receptor for IP10/Mig (CXCR3, Loetscher, M., Gerber, B., Loetscher, P., Jones, S.A., Piali, L., Clark-Lewis, I., Baggiolini, M., and Moser, B. (1996) J. Exp. Med. 184, 963-969.);
a receptor for SDF-1 (CXCR4 or LESTR, Bleul, C.C., Farzan, M., Choe, H., Parolin, C., Clark-Lewis, I., Sodroski, J., Springer, T.A. (1996) Nature, 382, 829-836.)

In addition, the following human CC chemokine receptors:

MIP-1α/RANTES receptor (CCR-1, Neote, K., Digregorio, D., Mak,J.K., Horuk, R. and Schall, T.J. (1993) *Cell* 72, 415-425; Gao, B. J-L., Kuhns, D.B., Tiffany, H.L., McDermott, D., Li, X., Francke, U. and Murphy, P.M. (1993) *J. Exp. Med.* 177, 1421-1427);

MCP-1A and B receptors (CCR-2A and B, Charo, I.F., Myers, S.J., Herman, A., Franci, C., Connolly, A.J. and Coughlin, S.R. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 2752-2756; Yamagami, S., Tokuda, Y., Ishii, K., Tanaka, T. and Endo, N. (1994) *Biochem. Biophys. Res. Commun.* **202**, 1156-1162);

the eotaxin/RANTES receptor (CCR-3, Combadiere, C., Ahuja, S.K. and Murphy, P.M. (1995) *J. Biol. Chem.* **270**, 16491-16494; Daugherry, D.L., Siciliano, S.J., DeMartino, J.A., Malkowitz, L., Sirotina, A. and Springer, M.S. (1996) *J.Exp.Med.* **183**, 2349-2354; Kitaura, M., Nakajima, T., Imai, T., Harada, S., Combadiere, C., Tiffany, H.L., Murphy, P.M. and Yoshie, O. (1996) *J. Biol Chem.* **271**, 7725-7730),

the promiscuous receptor on basophils (CCR-4, Power, C.A., Meyer, A., Nemeth, K., Bacon, K.B., Hoogewerf, A.J., Proudfoot, A.E.I. and Wells, T.N.C. (1995) *J. Biol. Chem.* **270**, 19495-19500);

a new MIP-1α/MIP-1β/RANTES receptor (CCR-5, Samson, M., Labbe, O., Mollereau, C., Vassart, G. and Parmentier, M. (1996) *Biochemistry* **35**, 3362-3367.)

a new receptor for LARC (CCR6, Baba, M., Imai, T., Nishimura, M., Kakizaki, M., Takagi, S., Hieshima, Nomiyuki, H., and Yashie, O. (1997) J. Biol Chem. 272, 14893-14898.)

a new receptor for ELC/exodus3 (CCR7, Yoshida, R., Imai, T., Hieshima, K., Kusuda, J., Baba, M.; Kitaura, M., Nishimura, M., Kakizaki, M., Nomiyama, H., and Yoshie, O. (1997) J. Biol. Chem. 272, 13803-13809.)

a new receptor for I-309 (CCR8, Samson, M., Stordeur, P., Labbe, O., Soularue, P., Vassart, G., and Parmentier, M. (1997) Eur. J. Immunol. 26, 3021-3028; Tiffany, HL, Lautens, LL, Gao,J-L, Pease, J., Locati, M., Combadiere, C., Modi, W., Bonner, T.I. and Murphy, P.M. (1997) *J Exp. Med.* **186**, 165-170; Stuber-Roos, R., Loetscher, M., Legner, D.F., Clark-Lewis, I., Baggiolini, M. and Moser, B. (1997) J. Biol. Che. 272, 17251-17254)

Recently the receptor for the newly described CX3C chemokine fractalkine/neurotacin has also been identified (Imai, T., Hieshima, K., Haskell, C., Baba, M., Nagira, M., Nishimura, M., Kakizaki, M., Takagi, S., Nomiyama, H., Schall, T.J., Yoshie, O. (1997) Cell 91, 521-530.).

Chemokin receptors belong to the group of 7 transmembrane (7TM) spanning receptors and their signal transduction pathway involves pertussus toxin-sensitive G-protein and a rise in $[Ca^{2+}]_i$. Although details about the molecular events are still incomplete, a complex array of intracellular signals ultimately lead to leucocyte activation and chemotaxis (Premack, B.A. and Schall, T.J. (1996) Nature medicine 2, 1174-1178).

Chemokine receptors, like chemokines, are divided into at least three sub-families, the CXC chemokine receptors (CXCR), the CC chemokine receptors (CCR) and the CX3CR, based on their selectivity for either CXC, CC, CX3C chemokines. Ligand cross-selectivity, that is CXCRs that bind CC chemokines or *vice versa*, is not observed. Chemokine receptors consist of 350-368 amino acids and the sequence identity amongst members of the receptor sub-families is wide (36-77%). Most chemokine receptors recognise more than one chemokine and many chemokines, including IL-8, RANTES, MIP-1α and the MCPs, bind to more than one receptor (Roos *et al*, J Biol Chem, 1997, 272 (28), 17521).

Current research suggests a pathophysiological role for chemokines in wide range of inflammatory states and infectious diseases. These disease states include, but are not limited to various viral, bacterial and parasital infection, rheumatoid arthritis, atherosclerosis and restenosis, psoriasis, asthma, chronic contact dermatitis, inflammatory bowel disease, multiple sclerosis, stroke, sarcoidosis, idiopathic pulmanory fibrosis as well as organ transplant rejection. Chemokines and their receptors have been recognised as targets for therapeutic agents.

The orphan 7TM receptor, HBMBU14, has been identified using EST (Expressed Sequence Tag) sequencing technology (Adams, M.D., *et al. Science* (1991) 252:1651-1656; Adams, M.D. *et al.*, *Nature*, (1992) *355*:632-634; Adams, M.D., *et al.*, *Nature* (1995) 377 Supp:3-174) and the full-length cDNA isolated (EP application no. 97307428.9; SmithKline Beecham Corp.). The sequence alignment of HBMBU 14 shows 100% identity with the published 7TM receptors TYMSTR (T- lymphocyte expressed seven transmembrane domain receptor) (Loetscher, M., *et al*, 1997 Current Biology **17**, vol 9, 652-660), STRL-33, (Liao, F., *et al*, 1997, J. Exp. Med, **185**, no.11, 2015-2023), and BONZO (Deng, H., *et al*, 1997, Nature, **388**, 296-300).

TYMSTR is reported to be expressed in activated T-lymphocytes but not in freshly isolated lymphocytes and leucocytes. It has been identified as a co-receptor for human immunodeficiency virus -1 (HIV-1). Similarly, STRL33, has been reported to act as a co-receptor for both M-tropic and T-tropic HIV-1 (Liao, F., *et al* 1997, J. Exp Med **185**, 11, 2015-23), and also as a co-receptor for SIV (Nature 1997 **388**, 238). STRL33 has also been found to be a co-receptor for SIV as well as HIV-1 (Deng *et al*, Nature 1997, **388**, 296-300). BONZO mRNA has been detected in monocytes (Farzan, M., *et al*, 1997, J.Exp. Med **186**, no3, 405-411). It has also been reported that human peripheral blood mononuclear cells express an SIV co-receptor(s) that is distinct from CCR5 (Nature **238**, 388). No ligands have, to date, been identified for TYMSTR, STRL33 or BONZO.

The CXC protein, PF-4 (platelet factor -4), is a 70 residue polypeptide released from α- granules when platelets are activated by contact with collagen or other thrombotic stimuli. The most characterised activity of PF-4 is inhibition of the anticoagulant activity of heparin through blockade of its interaction with anti thrombin III and other coagulation factors, thereby promoting thrombosis at sites of platelet activation (Ruckinski B *et al*, 1979, Blood, **53**, 47-62). PF-4 also exhibits several inflammatory activities. It is chemoattractant for monocytes and neutrophils at concentrations found in human serum (Deuel, T *et al*, 1981 Proc Natl Acad Sci **78**, 7, 4584-87) and fibroblasts (Senior, R.M., *et al*, 1988 J.Cell Biol **96**, 382-385). This suggests a link between initiation of thrombosis and induction of inflammation, whilst the fibroblast recruitment would enable wound healing. The carboxy terminal tridecapeptide of PF-4 has been demonstrated to be a potent chemotactic agent for monocytes (Osterman D. *et al*, Biochem Biophys Res Commun 1982, **107**,1, 130-135). Goldman D.W. *et al* (1985, Immunology **54**, 163-172), have similarly reported the chemoattractant activity of PF-4 and a substituent dodecapeptiode, designated 59-70, towards monocytes and neutrophils. Human PF-4 has also been reported to reverse or block experimentally induced immunosuppression in mice (Barone, A.D., *et al*, 1988, J.Biol.Chem **263**, 8710-8715; Yin, J-Z., *et al*, 1988 Cell Immunol **115**, 221-227). PF-4 has been shown to inhibit angiogenesis by prevention of endothelial cell proliferation in response to growth factors (Maione, T.E., *et al*, 1990 Science **247**, 77-79). Van Damme *et al*, 1989, Eur J Immunol **12**, 2367-73 describe an additional monocyte chemotactic protein released from unfractionated cultured cells, and which they identify as PF-4. PF-4 has been shown to induce an acute local inflammatory response when dosed intradermally in the mouse (Sharpe, R.J. *et al* 1991, Cell Immunol. **137**, 1, 72-80). The inflammatory infiltrates consisted of neutrophils and mononuclear cells. Engstad C.S. *et al.* (1995 J. Leukoc. Biol. **56**, 5, 575-81) and Osterud, B. (1995 Blood

Coag Fibrinolysis 6 Suppl 1 520-25), suggest the involvement of PF-4 in enhanced LPS-stimulated monocyte tissue factor activity. Specific binding has been demonstrated for several PF-4 related peptides eg βTG, GRO, IL-8, but the identities of cell surface receptors are not known.

We have now demonstrated that PF-4 is a ligand for the identical receptors HBMBU14, TYMSTR (T- lymphocyte expressed seven transmembrane domain receptor), STRL-33, and BONZO. This receptor will be called hereinafter the PF-4 receptor. This identification of PF-4 as a ligand for the PF-4 receptor therefore facilitates the development of screening methods for identifying agonists and antagonists of the receptor.

In a first aspect, the present invention provides for a screening method for identifying antagonists of the PF-4 receptor which method comprises using the PF-4 receptor, suitably expressed on the surface of a host cell or in a membrane preparation, or as an isolated protein, in combination with the mature form of PF-4.

The PF-4 precursor has the nucleotide sequence given in SEQ ID NO:1 and the amino acid sequence given in SEQ ID NO:2. The amino acid sequence of mature PF-4 is given in SEQ ID NO:3.

PF-4 receptor has the nucleotide sequence given in SEQ ID NO:4 and the amino acid sequence given in SEQ ID NO:5.

The invention provides for a screening method involving the use of cells which naturally express the PF-4 receptor on their surface, for example human monocytes. Another aspect of the invention concerns the use of recombinantly expressed PF-4 receptor in, for example, mammalian cells (eg. CHO and RBL cells), yeast, insect cells including *Drosophila* spp. and bacterial cells (eg. *E. coli*). Transfection of cells with PF-4 receptor cDNA can be achieved using standard methods, as described in, for example, Sambrook et al, Molecular Cloning, A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory Press, New York (1989). Receptor expression may be transient or stable. Preferably, the expression is stable. More preferably a mammalian cell line is transfected with an expression vector comprising a nucleic acid sequence encoding the PF-4 receptor, and the cell line then cultured in a culture medium, such that the receptor is stably expressed on the surface of the cell.

The invention further provides for a screening technique which involves the use of cells which express the PF-4 receptor, for example human monocytes or transfected CHO or RBL cells, in a system which measures stimulation of a chemotactic response. The cells are incubated with an effective amount of a compound added to the culture medium used to propogate the transfected cells expressing the receptor. An effective amount is a concentration sufficient to block the binding of PF-4 to the receptor, or alternatively to facilitate an agonistic effect. Inhibition of a chemotactic response to PF-4 would indicate that the particular compound is a potential antagonist of the receptor ie. the compound inhibits activation of the receptor. Incubation of the compound with the cells resulting in a chemotactic response would suggest that the compound is an agonist i.e. activates the receptor to stimulate chemotaxis.

In another aspect of the invention a screening technique involves the use of a microphysiometer which measures extracellular pH changes reflecting intracellular pH changes upon receptor activation, as described in Science, (1989) 246, 181-296. on cells which express the PF-4 receptor. Incubation of the cells expressing the PF-4 receptor with potential agonist or antagonist compounds enable a second messenger response e.g. signal transduction or pH changes to be detected. In another aspect the invention makes use of a reporter gene system, for example using the luciferase gene. In this aspect receptor activation is linked to a detectable response, for example the emission of light in the luciferase system, which can be measured to determine the effectiveness of the potential agonist or antagonist.

In another aspect of the invention, mRNA encoding the PF-4 receptor is introduced into *Xenopus* oocytes or melanophores thereby facilitating transient expression of the receptor. The oocytes or melanophores when incubated with the compound to be screened together with PF-4 can be monitored for antagonist effects by an inhibition of a calcium or cAMP signal, or in the case of an agonist, by detection of stimulation of a calcium or cAMP signal.

A further aspect of the invention is a screening procedure whereby the PF-4 receptor is expressed on the surface of, for example, endothelial cells, smooth muscle cells or embryonic kidney cells, in which the receptor is linked to a phospholipase C or D. Screening for an agonist or antagonist involves the detection of activation of the receptor, or inhibition of activation of the receptor, from the phospholipase second signal.

Another screening technique of the invention involves the incubation of the compound with the cells expressing the PF-4 receptor (monocytes or transfected CHO or RBL cells) with subsequent detection of an induced calcium signal or the detection of an inhibition of a PF-4-stimulated calcium signal. This method provides a means for determining the compound's potential agonist or antagonist effect.

A further screening method of the invention involves screening for PF-4 receptor inhibition by determining the inhibition of binding of labelled PF-4 to whole cells (monocytes or transfected CHO or RBL cells) or to cell membranes which have the PF-4 receptor on their surface. This assay involves incubation of the cells with compounds in the presence of a labelled form of PF-4 e.g. radiolabelled with ^{125}Iodine. The amount of labelled ligand bound to the receptors is measured. If the compound is an antagonist the binding of labelled ligand to the receptor is inhibited.

The invention also provides for another method for screening for PF-4 inhibitors by measuring the effects on cAMP. When the PF-4 receptor is coupled to a stimulatory Gs protein PF-4 will increase cAMP levels. Eukaryotic cells expressing the PF-4 receptor, preferably transfected CHO or RBL cells, are exposed to compounds in the presence of PF-4, and reduction of cAMP accumulation is measured. In an alternative screen PF-4 signalling via its receptor is coupled to an inhibitory Gi protein. After, for example, a forskolin induced increase of cAMP, incubation with PF4 will lead to a PF4 receptor coupled reduction of cAMP levels. PF4 antagonists can be detected by measuring the prevention of this cAMP reduction.

Alternatively, an immunoassay may be used to detect PF-4 binding to its receptor by detecting the immunological reactivity of PF-4 with anti-PF-4 antibodies in the presence or absence of the test compound. The immunoassay may for example involve an antibody sandwich or an enzyme linked immunoassay (ELISA). Such methods are well known in the art and described in, for instance, Methods in Enzymology (1987, vol 154 and 155, Wu and Grossman, and Wu) and Methods in Cell and Molecular Biology (Academic Press, London).

This invention also provides a method for identifying other ligands for the PF-4 receptor, by using, for example, a standard radioligand competition assay whereby either PF-4 or the test ligand is labelled. A resulting competition assay with non-radiolabelled PF-4 or test ligand would provide a receptor affinity constant.

The systems described for identifying agonists and / or antagonists may also be used to determine ligands which bind to the receptor.

Agonists and/or antagonists may be identified from a variety of sources, for instance, from cells, cell-free preparations, chemical libraries and natural product mixtures. Such agonists and/or antagonists may be natural or modified substrates, ligands, receptors, enzymes, etc., as the case may be, of PF-4; or may be structural or functional mimetics of the polypeptide of the present invention. See Coligan *et al.*, *Current Protocols in Immunology* 1(2):Chapter 5 (1991). Potential PF-4 receptor antagonists may include an antibody, or an oligonucleotide, which binds to the G-protein coupled receptor, but does not induce a second messenger response, such that the activity of the G-protein coupled receptor is prevented. Other potential antagonists include small molecules, e.g. small peptides or peptide-like molecules or organic molecules, which bind to the PF-4 receptor, making it inaccessible to ligands, such that normal biological activity is prevented. Potential antagonists may also include proteins that are closely related to the ligand of the PF-4 receptor, i.e. a fragment of the ligand, which have lost biological function, and which, when binding to the PF-4 receptor, elicit no response.

Another potential antagonist is a soluble form of the PF-4 receptor, for example a fragment of the receptor, which binds to PF-4 and prevents PF-4 from interacting with the membrane bound PF-4 receptor.

Compounds identified using the screen will be of use in therapy. Accordingly, in a further aspect, the present invention provides a compound identified as an agonist or an antagonist of the PF-4 receptor for use in therapy.

Agonists for PF-4 receptors may be used for therapeutic purposes, such as treatment for HIV, AIDS, rheumatoid arthritis, asthma, psoriasis, atherosclerosis and other inflammatory diseases.

Antagonists for PF-4 receptors may be used for therapeutic purposes, including but not limited to treatment for HIV, AIDS, rheumatoid arthritis, asthma, psoriasis, atherosclerosis and other inflammatory diseases.

Accordingly, in a further aspect, this invention provides a method of treating an abnormal condition related to an excess of PF-4 receptor activity and/or a ligand thereof, for example PF-4, which comprises administering to a patient in need thereof an antagonist as hereinbefore described in an amount effective to block binding of ligands to the receptor, or by inhibiting a second signal, and thereby alleviating the abnormal conditions.

This invention also provides a method of treating an abnormal condition related to an under-expression of PF-4 receptor activity and/or a ligand thereof which comprises administering to a patient in need thereof a therapeutically effective amount of an agonist compound which activates the receptor as hereinbefore described and thereby alleviate the abnormal conditions.

Identification of a ligand for the PF-4 receptor, such as PF-4, allows for the effective identification of polyclonal or monoclonal antibodies raised against the PF-4 receptor which are neutralising antibodies. Such neutralising antibodies are of use in therapy, in comparison to non-neutralising antibodies which are ineffective. Accordingly, in a further aspect, the present invention provides for the use of neutralising antibodies raised against the PF-4 receptor in therapy.

Such antibodies may be, for example, polyclonal or monoclonal antibodies. The present invention also includes chimeric, single chain or humanised antibodies, as well as Fab fragments, or the product of an Fab expression library. Various procedures well known in the art may be used for the production of such antibodies.

Antibodies generated against the PF-4 receptor may be obtained by direct injection of the isolated receptor into an animal or by administering the receptor to an animal, preferably a non-human. The antibody so obtained will then bind the receptor. For preparation of monoclonal antibodies, any technique which provides antibodies produced by continuous cell line cultures may be used, for instance the hybridoma technique (Kohler and Milstein, 1975, Nature, 256:495-497), the trioma technique, the humanB-cell hybridoma technique (Kozbor et al, 1985, Immunology Today, 4:72) and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al, in Monoclonal Antibodies and Cancer Therapy, Alan R Liss Inc, 1975: 77-96). Techniques described for the production of single chain antibodies in US 4,946,778 can be adapted to produce single chain antibodies to immunogenic polypeptides. In addition, transgenic mice may be used to express humanised antibodies to immunogenic polypeptides.

Compounds, including antibodies. for use in such methods of treatment will normally be provided in pharmaceutical compositions. Accordingly, in a further aspect. the present invention provides for a pharmaceutical composition comprising a compound identified as an inhibitor or an activator of the PF-4 receptor and a pharmaceutically acceptable excipient or carrier. The PF-4 agonists or antagonists may be administered in combination with a suitable pharmaceutical carrier e.g. saline, buffered saline, dextrose, water, glycerol, ethanol, or combinations of these agents. The formulation consists of a therapeutically effective amount of the agonist or antagonist, and the pharmaceutically acceptable carrier.

The formulations may be administered by topical, intravenous, intraperitoneal, intramuscular, intranasal or intradermal routes, in amounts which are effective for treating and / or prophylaxis of the specific indication. In general, the formulations will be administered in an amount of at least 10ug/kg body weight, and not in excess of about 8mg/kg body weight. In most cases, the dosage is from about 10μg/kg to about 1mg/kg body weight daily, depending on the route of administration, symptoms etc.

Compounds which are active when given orally can be formulated as liquids, for example syrups, suspensions or emulsions, tablets, capsules and, lozenges.

A liquid formulation will generally consist of a suspension or solution of the compound or pharmaceutically acceptable salt in a suitable liquid carrier(s) for example, ethanol, glycerine, non-aqueous solvent, for example polyethylene glycol, oils, or water with a suspending agent, preservative, flavouring or colouring agent.

A composition in the form of a tablet can be prepared using any suitable pharmaceutical carrier(s) routinely used for preparing solid formulations. Examples of such carriers include magnesium stearate, starch, lactose, sucrose and cellulose.

A composition in the form of a capsule can be prepared using routine encapsulation procedures. For example, pellets containing the active ingredient can be prepared using standard carriers and then filled into a hard gelatin capsule; alternatively, a dispersion or suspension can be prepared using any suitable pharmaceutical carrier(s), for example aqueous gums, celluloses, silicates or oils and the dispersion or suspension then filled into a soft gelatin capsule.

Typical parenteral compositions consist of a solution or suspension of the compound or pharmaceutically acceptable salt in a sterile aqueous carrier or parenterally acceptable oil, for example polyethylene glycol, polyvinyl pyrrolidone, lecithin, arachis oil or sesame oil. Alternatively, the solution can be lyophilised and then reconstituted with a suitable solvent just prior to administration.

A typical suppository formulation comprises an active compound or a pharmaceutically acceptable salt thereof which is active when administered in this way, with a binding and/or lubricating agent such as polymeric glycols, gelatins or cocoa butter or other low melting vegetable or synthetic waxes or fats.

Preferably the composition is in unit dose form such as a tablet or capsule.

Each dosage unit for oral administration contains preferably from 1 to 250 mg (and for parenteral administration contains preferably from 0.1 to 25 mg) of an inhibitor of the invention.

The daily dosage regimen for an adult patient may be, for example, an oral dose of between 1 mg and 500 mg, preferably between 1 mg and 250 mg, or an intravenous, subcutaneous, or intramuscular dose of between 0.1 mg and 100 mg, preferably between 0.1 mg and 25 mg, of the active compound, or a pharmaceutically acceptable salt thereof, calculated as the free base, the compound being administered 1 to 4 times per day. Suitably the compounds will be administered for a period of continuous therapy.

Identification of a ligand for the PF-4 receptor provides a means of developing a diagnostic assay for measuring levels of PF-4 receptor in a patient. Accordingly, in a further aspect, the present invention provides for a diagnostic assay for detecting diseases or susceptibility to diseases related to abnormal activity of the PF-4 receptor. Assays used to detect levels of PF-4 in a sample derived from a patient are well known in the art and include radioimmnuoassays, Western blot analysis and ELISA assays. The invention also provides a diagnostic kit comprising PF-4.

The invention is further described in the following examples which are intended to illustrate the invention without limiting its scope. In order to facilitate understanding of the following examples certain methods and/or terms will be described.

"5' and 3' untranslated sequences" refer to those sequences in mRNA flanking the protein coding region and which themselves do not code for protein. In eukaryotic mRNA the 5' and 3' untranslated region are of variable length, the 3' untranslated sequences frequently reaching several kilobases in length. It is usual to refer to these terms in the context of the corresponding DNA sequences for convenience.

"Kozak" sequence refers to a consensus nucleotide sequence surrounding the AUG start codon of a protein encoding mRNA, usually in eukaryotic organisms (see for example Kozak, M., *Nucleic Acids Res* (1984) May 11;**12(9)**:3873-3893). In the present invention this sequence may be natural, ie. identified from a naturally occurring mRNA source, or synthetic, the latter being designed either by experimentation or in accordance with 'rules' known in the art. It is usual to refer to this term in the context of the corresponding DNA sequences for convenience.

All publications, including but not limited to patents and patent applications, cited in this specification are herein incorporated by reference as if each individual publication were specifically and individually indicated to be incorporated by reference herein as though fully set forth.

Examples

1. Cloning of recombinant human PF-4 receptor

An EST (Expressed Sequence Tag), HBMBU14, with 37 % homology to CC-CKR-2B receptor (Charo, I.F., Myers, S.J., Herman, A., Franci, C., Connolly, A.J. and Coughlin, S.R. (1994) *Proc. Natl. Acad. Sci. USA* **91**, 2752-2756) was identified from the HGS (Human Genome Sciences) database. The corresponding cDNA clone was isolated and the entire cDNA insert sequenced. Sequence analysis showed that this fragment contained only a portion of the 3' coding region. In order to obtain the full-length sequence, the HBMBU14 cDNA partial fragment was amplified by PCR using the primers:

1) 5' CTA CAC CAT CCA TGG TGA CAG

2) 5' TCA AGC ATT CCA TGG ACC AC

and the product was radiolabelled and used to screen the human PAC genomic library using hybridization techniques well known in the art (see for example Sambrook et al., MOLECULAR CLONING, A LABORATORY MANUAL, 2nd Ed.; Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989). A positive clone was identified and sequenced. Sequence analysis showed that in the corresponding region the genomic sequence is identical to the EST sequence. Primers corresponding to the 3' coding region of the HBMBU14 were designed and used to sequence the upstream coding region of the clone. The full length cDNA clone encodes 342 amino acids and which has about 38.5% identity in 342 amino acid residues with the CC-CKR-2B receptor and 34% identity in 295 amino acid residues with the human MCP-1 receptor (GENBANK Accession No. U03905).

The cDNA clone was subcloned into the mammalian expression vector pCDN (Aiyar, N., Baker, E., Wu, H.-L., Nambi, P., Edwards, R. M., Trill, J. T., Ellis, C. and Bergsma, D. J. *Mol. Cell Biochem.* **131**:75-86, 1994) by PCR using PAC genomic DNA as the template and with the following primers:

1) 5' Primer 5' TC AG*A AGC TT*C ACC ATG GCA GAG CAT GAT TA

HindIII Translation start

2) 3' Primer 5' TCT C*GG ATC C*CT GGC AAG GCC **TA**T AAC TGG AAC ATG CTG

BamHI Translation stop

The primers were designed to remove all 5' and 3' untranslated regions (UTRs) and to introduce useful restriction sites to aid subsequent subcloning of this PCR fragment into the pCDN vector; HindIII and BamHI flanking the coding region to the 5' and 3' respectively. Primer (1) also introduced a consensus Kozak sequence (underlined) in order to maximise expression from this plasmid construct. The sequences of the HBMBU14 coding region and the flanking PCR primers in the resultant expression construct were confirmed.

2. Stable expression of PF-4 receptor in mammalian cell lines

The pCDN vector carries both G418 resistance and dihydrofolate reductase genes for selection and amplification.

Stable cell lines of PF-4 receptor in CHO and RBL-2H3 cells were obtained by electroporation of the pCDN:PF-4 receptor vector followed by clonal selection using G418 at 400ug/ml in the growth medium. After 3 weeks in the selection medium individual clones were picked and expanded for further analysis.

A HEK293 stable cell line of PF-4 receptor was obtained by calcium phosphate precipitation followed by clonal selection using G418 at 400ug/ml as described above.

The CHO, RBL-2H3 and HEK293 lines were screened for high level receptor expression by Northern blot analysis using RNA isolated from the G418 lines and as hybridization probe the coding sequence of PF-4 receptor. From this screen, the clonal cell lines producting the highest PF-4 receptor RNA levels were chosen for futher study.

3. Monocyte isolation

Human peripheral blood monocytes are prepared from the blood of normal healthy volunteers, essentially as described by Boyum (*Methods in Enzymol*ogy (Academic Press, New York and London) **108**, 88-102). Blood is collected into anticoagulant (one part 50mM EDTA, pH 7.4, to nine parts blood), then centrifuged for 5 minutes at 600*g*. The upper layer of platelet-rich plasma

is removed and centrifuged for 15 minutes at 900*g*, to pellet the platelets. The upper layer of platelet-poor plasma is removed and added back to the packed red cells; the pelleted platelets are discarded. Dextran T500 is added (10 volumes EDTA blood to one volume 6% (w/v) dextran in 0.9% (w/v) NaCl) and the erythrocytes are allowed to sediment at unit gravity for 30 minutes. The resultant leukocyte-rich plasma is removed and centrifuged for 5 minutes at 400*g*. The cell pellet is resuspended in 5ml of the supernatant, and the suspension is underlayered with 3ml NycoPrep, then centrifuged for 15 minutes at 600*g*. The mononuclear layer at the interface between the plasma and the NycoPrep isremoved and washed through PBS by centrifugation for 5 minutes at 400*g*. The mononuclear layer typically contained ≥ 80% monocytes, determined by staining cytocentrifuge preparations for non-specific esterase using α-naphthyl-butyrate. Cell viability (typically >95%) is assessed as the ability to exclude trypan blue.

4. Ligand binding studies with receptor

a. Whole cell assay

^{125}I- labelled PF-4 was incubated with 1-2 x 10^6 cells (monocytes or transfected RBL or transfected CHO) in the presence or absence of increasing concentrations of unlabelled PF-4 for 15 minutes at 37°C in a 96 well plate. Following incubation, the cells were washed and collected onto a polyethylenimine-treated Whatman GF/C filter, using a Brandell cell harvester. Filters were washed and radioactivity bound to the filters was counted in a γ-counter. Fig 1A shows the binding results of RBL cells transfected with the PF-4 receptor and Fig 1B the results with PF-4 receptor transfected CHO cells.

b. Cell membrane assay (using membranes from transfected RBL or CHO cells)

^{125}I- labelled PF-4 was incubated with 50μg of membrane suspension in the presence or absence of increasing concentrations of unlabelled PF-4 for 2 hours at room temperature in a 96-well plate. Following incubation, the membranes were washed and collected onto a polyethylenimine-treated Whatman GF/C filter, using a Packard harvester. The plate was oven dried and radioactivity bound to the filter plate was counted in a γ-counter.

5. Functional studies with receptor

a. Chemotaxis

The chemoattractant activity of PF-4 towards RBL or CHO cells transfected with PF-4 receptor was monitored using a 96-well chemotaxis assay (see Figure 2). RBL or CHO cells transfected with an unrelated orphan chemokine receptor were used as controls and did not respond to PF-4. Briefly, cells which have migrated across a polycarbonate filter with pore size 5μm (RBL cells) or 8μm (CHO cells) towards the PF-4 (0 - 30nM), following incubation at 37°C and 5% CO_2 humidified air, were quantified colorimetrically from a standard curve relating cell density to absorbance at 590nm. The colorimetric end point derives from cellular reduction of 3-[4,5,dimethylthiazol-2-yl]-2,5, diphenyltetrazolium bromide from its formazan product.

The chemoattractant activity of PF-4 towards freshly isolated human monocytes was determined using a 48-well modified Boyden microchemotaxis chamber. Numbers of cells migrating across a polycarbonate filter (5μm pore size) following incubation at 37°C and 5% CO_2 humidified air were quantified by light microscopy of Diff-Quik stained filters, using a x40 objective and x10 ocular containing a 10mm^2 counting grid (Figure 3).

b. Calcium mobilisation

The ability of PF-4 or other chemokines to elicit a calcium signal in monocytes or cells transfected with the PF-4 receptor to elicit a calcium signal can be monitored by measuring the increased fluorescence of fura-2 or fluo-3.

6. mRNA expression in Different Cell Types

PolyA RNA was isolated from various cell types using guanidinum thiocyanate acid-phenol method. Poly A RNA is isolated using oligo dT column. RNA dot blot analysis is performed with a template manifold apparatus (Schleicher & Schuell, Keene, NH) to assure uniform dot size. Poly A RNA is applied using 0.5 ug of RNA. The RNA samples are denatured by adjusting them to 1M formaldehyde and heating them to 55°C for 15 min. The samples are diluted into 20 volumes of 3 M NaCl containing 0.3 M trisodium citrate and applied to nitrocellulose filters under a gentle vacuum. The filters are washed with additional diluted, baked at 80°C for 2h and then hybridized under high strengency in 50% formamide, 5 X SSPE, 5 X Denhardt's reagent,

0.1% SDS, and 100ug/ml yeast tRNA. The blots are washed with 0.1 X SSC, 0.1% SDS at 50°C and exposed to X-ray film for 48h at -70°C. Quantitation of the dots is performed using Phospho-Imaging analysis.

Poly A RNA was isolated from monocytes, macrophages, foam cells, MonoMac, Thp1, PBL, EOL3, neutrophils, T-cells, SM cells and endothelial cells. Relatively high level of expression was observed in monocytes, monomac, THP1, and PBL cells. Low level of expression was observed in EOL3 and endothelial cells.

7. Membrane Preparation and High Throughput Screen for the PF-4 Receptor

For the discovery of antagonists and agonists of the PF-4 receptor, a PF-4 binding competition assay is most useful. As a source of the PF-4 receptor, CHO or HEK 293 cells, stably transfected with the PF-4 receptor, could be used although other cells transfected with the PF-4 receptor or cells that naturally show a high level expression of the PF-4 receptor could also be employed. Typically the culture of cells expressing the PF-4 receptor (see example 1) is scaled up to 30L and cells are recovered by centrifugation at 600 x g for 10 min. The cell pellet is then frozen in liquid nitrogen. Pellets usually contain around 10^9 cells. For membrane isolation, pellets are freeze/ thawed 3 times. They are then resuspended in ice cold 10mM Tris (pH 7.5), 1 mM EDTA (sodium salt) (40 mls/1e8 cells) and homogenized using a Dounce (glass/glass) homogenizer (20-25 strokes), followed by a Polytron suspension with 3--10 sec pulses on a 3/4 setting (Brinkman tissue homogenizer). This suspension is centrifuged at 300 x g for 10 min. Pellet is discarded and the supernatent fraction is centrifuged at 40,000 x g (Sorvall SS-34: 18,000 rpm) for 30 min. at 4 °C. Pellet is resuspended in homogenizing buffer using the polytron and washed one time. The pellet is resuspended in assay buffer (50 mM Tris pH 7.5) at a concentration of 1 - 4 mg protein/ml.

Membranes obtained this way are suitable for the set-up of a high throughput PF-4 binding competition assay to search for compounds that interfere in the ligand-receptor interaction. The total binding of PF-4 to these membranes is first tested to be linear with the amount of membranes used. The time period to reach equilibrium binding at a suitable temperature is also established and is in our experience about 1 h at a temperature of 20 °C. For the screening assay typically 25 µg of membrane protein per well is used in a total volume of 100 µl buffer containing 50 mM HEPES, 1 mM $CaCl_2$, 5 mM $MgCl_2$ and 0.5 % Bovine serum albumin (western blot quality), pH 7.4. The concentration of ^{125}I-PF-4 is typically 1-2 nM and 75.000

cpm /well. Specific binding of labeled PF-4 should be displaced completely by unlabeled PF-4 at concentrations of 100 nM or more. The compounds to be tested are typically disolved and added in DMSO and final concentrations of DMSO in the assay are 1 % or less. After incubation the contents of the wells are harvested on a polyethyleneimine-treated GF/C filter using a 96 well plate cell harvester and the filters is washed four times with typically 1 ml icecold wash buffer containing 20 mM HEPES 0.5M NaCl pH 7.4. To determine any antagonists of labelled PF-4 binding, the filters are counted.

Alternatively, PF4 could be tagged with either a fluorescent label (page 415-421, Handbook of Fluorescent Probes and Research Chemicals 6th Edition, RP Haugland editor, Molecular Probes), a biotin label or a enzyme tag like the alkaline phosphatase SEAP-tag (Hishiema,K. et al, J Biol Chem. 1997, 272, 5846-53). The binding assay itself would typically be very similar to that described for radiolabeled PF4. Detection of bound PF4 would be by measuring fluorescence, binding of streptavadin or by measuring enzyme activity. As an alternative for detection of ^{125}I-PF4 by filter binding a SPA assay (Amersham) might be used.

Active compounds are further evaluated for their effect on ligand (PF-4) induced transient increases in intracellular calcium concentration. This assay is also able to distinguish whether compounds identified by the membrane binding assay are antagonists or agonists.

For measurements of cytosolic Ca^{2+} concentrations, appropriate cell lines transfected with the PF-4 receptor should be used. Appropriate cells include CHO cells and HEK 293 cells, if required co-transfected with appropriate G -coupling proteins. For the intracellular calcium concentration measurements cells are incubated with 0.5 µM FURA-2AM for 30 minutes at 37°C in HEPES-buffered saline (145mM NaCl, 5mM KCl, 1mM $MgCl_2$, 10mM HEPES and 10mM glucose), pH 7.4 at 37°C, supplemented with 1% albumin (w/v) and 1mM $CaCl_2$. After loading with FURA-2 the cells are centrifuged for 5 minutes at 300*g*, then resuspended in buffer containing no added albumin, to a cell density of 1.5 x 10^6 cells /ml, and kept at room temperature until use. Typically, this protocol results in a cytosolic FURA-2 concentration of approx. 100µM. Serial dilutions of chemokines in PBS + 0.1% albumin (w/v) - sterile-filtered - are added to aliquots (0.7ml) of cell suspension. FURA-2 fluorescence is measured at 37°C in a single excitation, single emission (500nm) wavelength Perkin Elmer LS5 fluorimeter. $[Ca^{2+}]_i$ are calculated from changes in fluorescence measured at a single excitation wavelength of 340nm. as described by Grynkiewicz.G.. Poenie, M.,& Tsien, R.Y. (1985) *J. Biol. Chem.* **260**,

3440-3450. Compound are normally added to the cells in DMSO solutions (final concentration less than 0.2%) and if compounds have agonist properties an effect on $[Ca^{2+}]_i$ is observed. Receptor function antagonism is evident if compounds are able depress the signal induced by the subsequent addition of PF-4 at a concentration shown to give a 75% maximal calcium signal in the absence of any compound.

The effects of compounds on PF-4 binding and response in physiologically relevant cells expressing the PF-4 receptor are normally tested in freshly isolated peripheral blood monocytes or peripheral blood lymphocytes.

Claims

1. A screening method for identifying antagonists of the PF-4 receptor which method comprises' using the PF-4 receptor in combination with PF-4.

2. A method as claimed in claim 1 in which the PF-4 receptor is expressed on the surface of a host cell or in a membrane preparation.

3. A method as claimed in claim 1 in which the PF-4 receptor is used in the form of the isolated protein.

4. A method as claimed in claim 1 which comprises the initial steps of expressing and isolating recombinant PF-4 receptors, and/or their extracellular domains.

5. A method as claimed in claim 4 in which a mammalian cell line is transfected with an expression vector comprising a nucleic acid sequence encoding the PF-4 receptor, and the cell line then cultured in a culture medium, such that the receptor, and/or their extracellular domain is stably expressed.

6. A method as claimed in claim 5 in which an antagonist is identified by adding an effective amount of a compound to the culture medium used to propagate the transfected cells expressing the receptor and then measuring the loss of binding of PF-4.

7. A method as claimed in claim 6 in which the PF-4 is labelled using a radiolabel, a biotin, enzyme or a fluorogenic label and the amount of labelled PF-4 bound by the receptor is measured in the presence and absence of the test compound.

8. A method as claimed in claim 6 in which the response of a known second messenger system is measured in the presence or absence of test compound.

9. A method as claimed in claim 6 in which the level of a functional response is measured in the presence or absence of test compound.

10. A method as claimed in claim 6 which involves the the use of melanophores which are transfected to express the receptor; *Xenopus* oocytes in which the receptor is transiently expressed; or systems in which the receptor is linked to a phospholipase C or D.

11. A screening method for identifying agonists of the PF-4 receptor which method comprises contacting a compound with PF-4 receptor and measuring the change in a functional response or a second messenger system associated with the receptor.

12. A method as claimed in claim 11 in which the PF-4 receptor is expressed on the surface of a host cell or in a membrane preparation

13. The use of PF-4 in a screening method for identifying an antagonist of the PF-4 receptor which method involves the use of PF-4 which may be labelled or unlabelled.

14. A compound identified by any one of the screening methods defined in any one of the preceding claims for use in therapy.

15. A method of treating an abnormal condition related to an excess of PF-4 receptor activity and/or an excess of a ligand thereof which comprises administering to a patient in need thereof a therapeutically effective amount of an antagonist of the receptor identified using any one of the screening methods defined in any one of the claims 1 to 10.

16. A method of treating an abnormal condition related to an under-expression of PF-4 receptor activity which comprises administering to a patient in need thereof a therapeutically effective amount of an agonist compound using the screening method defined in claim 11 .

17. A pharmaceutical composition comprising a compound identified by any one of the screening methods defined in any one claims 1 to 13 and a pharmaceutically acceptable excipient or carrier.

18. A method of diagnosising susceptibility to disease states associated with abnormal expression of the PF-4 receptor which method comprises measuring the level of PF-4 receptor in a sample taken from a patient.

19. The use of PF-4 to identify neutralising or activating antibodies to the PF-4 receptor.

20. Antibodies as defined in claim 19 for use in therapy.

21. The use of PF-4 and the PF-4 receptor to identify further chemokine ligands for the receptor. using a competitive binding assay and labelled PF-4.

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Sequence Information

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Amino acid sequence of the PF-4 receptor

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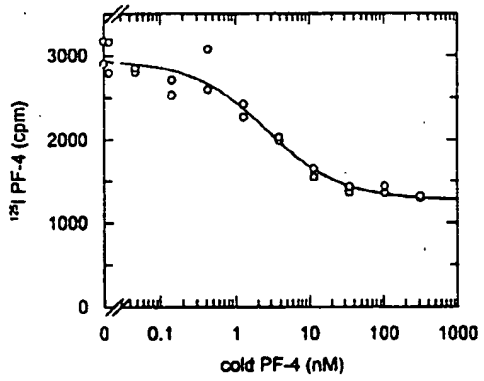
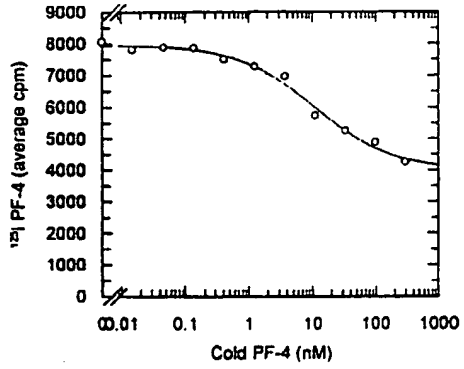
Figure 1

Binding of ^{125}I labelled PF-4 to RBL & CHO cells transfected with PF-4 receptor



A. RBL: IC_{50} = 2.7nM, Kd = 1.7



B. CHO: IC_{50} = 11.1nM, Kd = 19.3

Figure 2

PF-4-stimulated chemotaxis in PF-4 receptor-transfected RBL and CHO cells

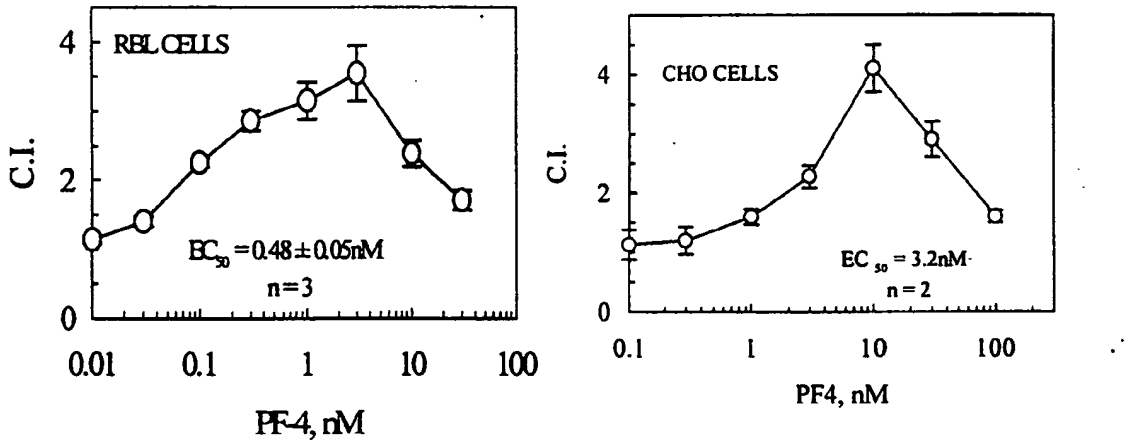
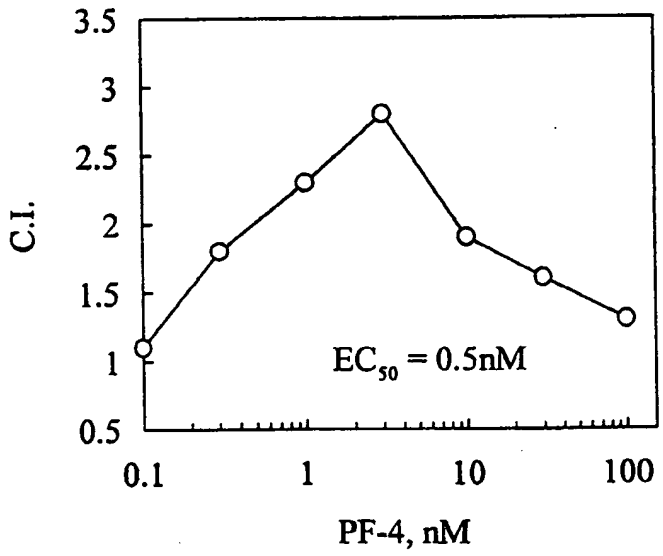


Figure 3

PF-4-stimulated chemotaxis in human monocytes



INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/GB 99/00950

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 G01N33/566 G01N33/86 C07K14/52 C07K16/28
C07K14/715

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ¹	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 28931 A (GENENTECH INCORPORATED) 22 December 1994 (1994-12-22) claims 1-17	14-20
A	WO 92 17497 A (GENENTECH INCORPORATED) 15 October 1992 (1992-10-15) claims 1-20; examples 1,2	1-21

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 August 1999	08/09/1999

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl. Fax: (+31-70) 340-3016	Van Bohemen, C

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INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern. nal Application No
PCT/GB 99/00950

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9428931 A	22-12-1994	US 5543503 A	06-08-1996
		US 5840856 A	24-11-1998
		US 5776457 A	07-07-1998
		US 5922541 A	13-07-1999
		US 5919896 A	06-07-1999
		US 5892017 A	06-04-1999
		US 5874543 A	23-02-1999
WO 9217497 A	15-10-1992	CA 2105998 A	30-09-1992
		EP 0577752 A	12-01-1994
		JP 6506697 T	28-07-1994
		US 5543503 A	06-08-1996
		US 5440021 A	08-08-1995
		US 5840856 A	24-11-1998
		US 5552284 A	03-09-1996
		US 5767063 A	16-06-1998
		US 5783415 A	21-07-1998
		US 5571702 A	05-11-1996
		US 5856457 A	05-01-1999
		US 5633141 A	27-05-1997
		US 5776457 A	07-07-1998
		US 5922541 A	13-07-1999
		US 5919896 A	06-07-1999
		US 5892017 A	06-04-1999
		US 5874543 A	23-02-1999